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Mouse *Dispatched homolog1* Is Required for Long-Range, but Not Juxtacrine, Hh Signaling

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Summary

Precise patterning of cell types along the dorsal-ventral axis of the spinal cord is essential to establish functional neural circuits [1]. In order to prove the feasibility of studying a single biological process through random mutagenesis in the mouse, we have identified recessive ENU-induced mutations in six genes that prevent normal specification of ventral cell types in the spinal cord. We positionally cloned the genes responsible for two of the mutant phenotypes, *smoothened* and *dispatched*, which are homologs of *Drosophila* Hh pathway components. The *Dispatched homolog1* (*Disp1*) mutation causes lethality at midgestation and prevents specification of ventral cell types in the neural tube, a phenotype identical to the *Smoothened* (*Smo*) null phenotype. As in *Drosophila*, mouse *Disp1* is required to move Shh away from the site of synthesis. Despite the existence of a second mouse *disp* homolog, Disp1 is essential for long-range signaling by both Shh and Ihh ligands. Our data indicate that Shh signaling is required within the notochord to maintain Shh expression and to prevent notochord degeneration. Disp1, unlike Smo, is not required for this juxtacrine signaling by Shh.

Results and Discussion

In an ongoing genome-wide screen for recessive ENU-induced mutations that affect the morphology of the midgestation mouse embryo, we identified six mutations that caused abnormal neural tube morphology and abnormal left-right asymmetry at e9.5. Among the mutants, *bent body* (*bnb*) [2] and *icbins* (*icb*) stood out because their phenotypes were so similar (Figures 1 and 2). Both arrested at e9.0–9.5, after initiating embryonic turning in a randomized direction. The embryos lacked asymmetrical heart looping and showed pericardial edema, suggesting that circulatory system defects were the cause of death. At the time of arrest, *bnb* and *icb* embryos had abnormally shaped heads and relatively large forelimbs.

Based on the lack of left-right asymmetry and the absence of a morphological floor plate at e8.5, we suspected that *bnb* and *icb* embryos failed to specify the floor plate and other ventral neural cell types. As predicted from the morphological observations, no expression of the floor plate marker Hnf3β was detected in the *bnb* or *icb* mutant neural tubes (Figures 1 and 2). Similarly, Shh expression was initiated in the notochord but was never detected in the ventral neural tube of the mutants (data not shown, and see below). Pax6, a marker of wild-type lateral neural cells, was expressed at high levels in an expanded domain including all lateral and ventral cells of the *bnb* and *icb* spinal cords. This is the same pattern of marker expression seen in *Shh* mutants at this stage [3]. Thus, there appeared to be no activity of the Shh pathway in the *bnb* and *icb* neural tubes.

We mapped *bnb* to a 2.2 cM (2.3 MB) interval on Chromosome 6 (Figure 1). This region included the mouse *Smo* gene, and a targeted null allele of mouse *Smo* causes a phenotype very similar to that of *bnb* [4]. Smo is a membrane protein that is required for the response to Hh [5–7]. Mouse mutants that lack *Smo* die earlier than *Shh* mutants, apparently because *Smo* is required for both Shh and Indian hedgehog (Ihh) signaling [4]. *bnb* mutant transcripts lack the normal 5′ coding exon of *Smo*, the same exon targeted in the *Smo* null allele (data not shown). We carried out complementation tests, which confirmed that *bnb* was allelic to *Smo*.

Both the *icb* and Smo^{bnb} phenotypes were more severe than the phenotype of *Shh* mutants [3], and they were indistinguishable from the phenotype of *Shh Ihh* double mutant embryos [4]. Thus, the *icb* mutation appeared to block the activity of both the Shh and Ihh pathways. We mapped *icb* to distal mouse Chromosome 1, where there are no previously defined mutations affecting the Hedgehog signaling pathway.

To define the step in the Shh pathway affected by *icb*, we analyzed the phenotype of embryos mutant for both *icb* and *Patched1* (*Ptch1*). Ptch1 is a membrane protein that binds Shh and is required to keep the downstream signaling pathway off in the absence of Hh ligand [8]. Both *Ptch1* and *icb* embryos arrested at e9.0–e9.5, but they did so with contrasting morphology; for example, the head of *Ptch1* embryos appeared truncated, and the neural tube was not closed, while the *icb* neural tube was closed (Figure 3). *icb Ptch1* double mutant embryos had the same morphology as *Ptch1* single mutant embryos, and this similarity suggests that the Shh targets are activated in *icb Ptch1* double mutants, as in *Ptch1* mutants. In the spinal cord, *Ptch1* and *icb* had opposite phenotypes. The *Ptch1* neural tubes were ventralized [9]: the floor plate marker *Hnf3*β was expressed across the neural plate, and the lateral neural marker *Pax6* was not expressed. The *icb* neural tubes were dorsalized: the floor plate marker *Hnf3*β was not expressed, and

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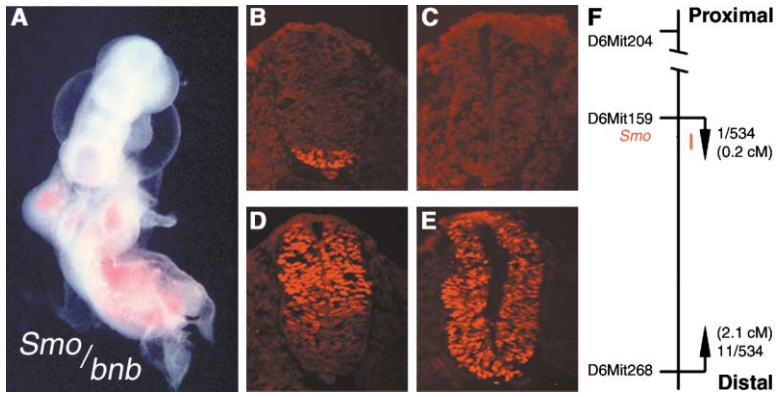

Figure 1. *bent body* Is an Allele of *Smo*

(A) *bnb* and *Smo* fail to complement. This *bnb*/*Smo* e9.5 embryo from a *bnb*/+ × *Smo*/+ intercross is indistinguishable from either *bnb*/*bnb* or *Smo*/*Smo* embryos at the same stage; we therefore renamed the ENU-induced mutation Smo^{bnb}.

(B–E) Immunohistochemical staining for (B and C) Hnf3β and (D and E) Pax6 in sections of the notochord and spinal cord in e9.5 (B and D) wild-type and (C and E) Smo^{bnb} embryos.

(F) Genetic mapping of the *bnb* interval on Chromosome 6. The number of recombination events per number of meiotic opportunities is indicated for the polymorphic markers D6Mit159 and D6Mit268. These markers lie 2.3 Mb apart.

the lateral neural marker *Pax6* was expressed in an expanded domain. The neural tissue of *icb Ptch1* double mutant embryos was ventralized, like the *Ptch1* single mutant: *Hnf3*β was expressed in all neural cells, and no *Pax6* expression was detected. Thus, all aspects of the *icb Ptch1* double mutant phenotype were indistinguishable from the *Ptch1* single mutant, and *icb* appears to act upstream of receptor Ptch1. Because Shh protein was present in the *icb* notochord, *icb* must act at a step between the production of Shh protein in the notochord and binding of Shh to the Ptch1 receptor in the ventral neural tube.

The nascent Hh protein undergoes an autocatalytic cleavage that covalently links a cholesterol group to a new carboxy terminus [10–12]. *Drosophila disp* encodes a transmembrane protein that is absolutely required for the secretion of the cholesterol-modified Hh but is not required for the secretion of a truncated form of Hh that cannot be modified [13, 14]. Two mouse genes show homology to *disp*. The more closely related homolog, which we called *Dispatched homolog1* (*Disp1*), mapped on Chromosome 1 in the interval that included *icb*; this evidence points to *Disp1* a candidate for the *icb* gene. *Disp1* was 22% identical and 14% similar to *Drosophila disp* and 85% identical to the human protein (MGC13130) that maps to the syntenic region on human Chromosome 1.

In contrast to *Drosophila disp*, which is expressed ubiquitously in embryos and imaginal discs [13], *Disp1* showed a restricted expression pattern (Figure 4). At e8.5–9.5, when *icb* embryos first showed abnormal morphology, *Disp1* was expressed in tissues that require Hh signaling, including the notochord, ventral neural tube, somites, branchial arches, and limb buds. The pattern of expression of *Disp1* was restricted by e10.5 to high levels in head mesenchyme, neural tube, somites, and limb ectoderm. While *Disp1* expression generally correlated with tissues known to possess active Hedgehog signaling, it was interesting to note that the *Disp1* expression became localized to the anterior half of the forelimb buds, away from the posterior source of Shh.

We identified additional dinucleotide repeat sequences that were polymorphic in our mapping cross, which made it possible to narrow the interval including *icb* to a 0.5-cM interval (Figure 2). *Disp1* lay at the center of this 1-MB region. We sequenced the 4.8-kb *Disp1* cDNA from *icb* mutant embryos and identified a G-to-T transversion mutation that changed a cysteine to a phenylalanine at amino acid 829 of the protein. Disp1 is a 1521 aa protein with 10–13 transmembrane domains, including a sterol sensing domain (SSD). Topology programs predict that C829 lies in a large extracellular loop just C-terminal to the SSD [15–18]. This extracellular

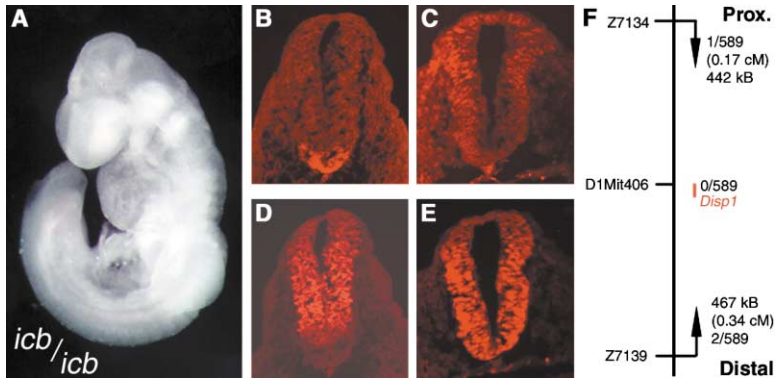

Figure 2. *icbins* Is an Allele of *Disp1*, a Sterol-Sensing Domain Protein

(A) The phenotype of an e9.5 *icb* embryo shows the striking resemblance to Smo^{bnb}.

(B–E) Immunohistochemical staining for (B and C) Hnf3β and (D and E) Pax6 in sections of the notochord and spinal cord in e9.5 (B and D) wild-type and (C and E) $Disp1^{icb}$ embryos.

(F) Genetic mapping of the *icb* interval on Chromosome 1. The number of recombination events per number of meiotic opportunities is indicated for the polymorphic markers as are the physical distances from the markers to *Disp1*. The newly identified markers flanking the *icb* interval are shown, and their sequences are: Z7139F (5′-CTTGCTTGTTCCTCCCATTC-3′), Z7139R (5′-TGTCTTGTCAACTGCCCATC-3′), Z7134F (5′-GCAGGCATGATAC CAAAAGG-3′), and Z7134R (5′-ACATCTGCCCTTCAGTGTCC-3′). *icb* never recombined with D1Mit406, a marker 4 kb upstream of the *Disp1* promoter.

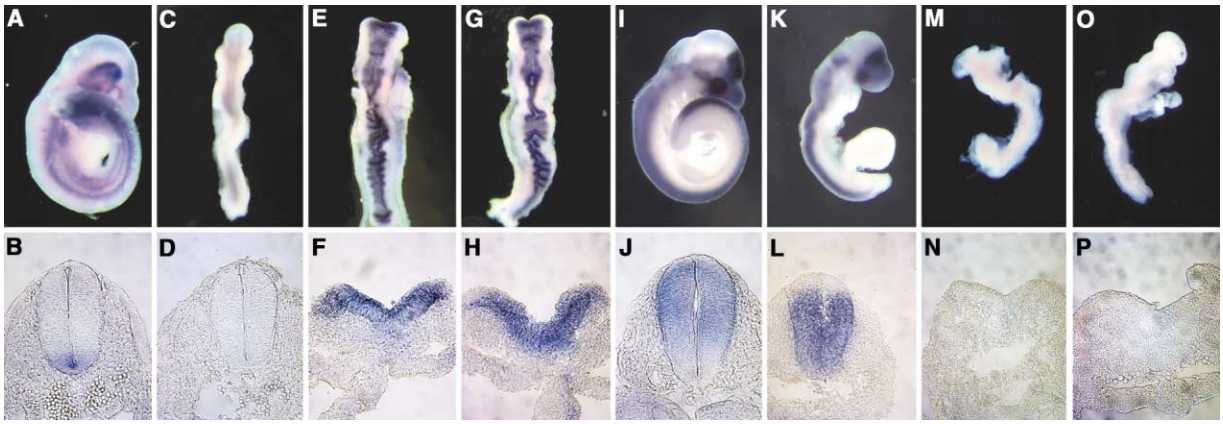

Figure 3. *Ptch1* Acts Downstream of *icb*

(A–P) Whole-mount in situ hybridization, with corresponding section beneath, of (A, B, I, and J) wild-type, (C, D, K, and L) *icb*, (E, F, M, and N) *Ptch1*, and (G, H, O, and P) *icb Ptch1* e9.5 embryos, showing expression of (A–H) *Hnf3*β and (I–P) *Pax6*. (E and M) *Ptch1* embryos are indistinguishable in morphology from (G and O) *icb Ptch1* embryos and are very different from (C and K) *icb* embryos. *Hnf3*β is expressed in the floor plate of (B) wild-type embryos, but it is not expressed in (D) *icb* embryos. *Hnf3*β is expressed across the neural plate of ventralized (F) *Ptch1* and (H) *icb Ptch1* embryos. *Pax6* is expressed in the lateral spinal cord of (J) wild-type embryos and in lateral and ventral neural cells in (L) *icb* embryos, but it is not expressed in (N) *Ptch1* or (P) *icb Ptch1* embryos.

loop in *Disp1* contains six cysteines, including C829, which are conserved in all *dispatched* family members (Figure 5). The cysteine-to-phenylalanine change in the *icb* mutation is likely to disrupt a disulfide bond and cause destabilization of the loop or the protein. Because this mutation should disrupt *Disp1* function and because *icb* blocks Shh signaling at a point between Shh and expression of Ptch1, the step likely to be affected by *Disp1*, we conclude that the missense mutation blocks most or all function of *Disp1* and is responsible for the *icb* phenotype. Thus, although there are two *Ptch* genes and two *Disp* genes in the mouse, it appears that, in both cases, only one of the homologous genes plays a central role in Hedgehog signaling [19, 20].

Drosophila disp is required in Hh-producing cells to allow release of active Hh, and clones of homozygous *disp* mutant cells in imaginal discs appear to accumulate high levels of Hh protein [13, 14]. We therefore examined the distribution of Shh in embryos at e9.5 by confocal microscopy (Figure 6). In wild-type embryos, Shh protein made in the notochord spreads to the ventral neural tube and then activates Shh expression in the floor plate [21]. High levels of Shh protein were present in the notochord cells of *Disp1*^{icb} embryos; however, there was no detectable Shh protein in the ventral neural tube. Thus, the Shh made in the *Disp1*^{icb} notochord fails to induce Shh expression in the ventral neural tube. Given that *Disp1*^{icb} acts upstream of *Patched*, the results suggest that mouse *Disp1*, like *Drosophila disp*, is required for the spread of Shh ligand from the cells where it is synthesized. Further, the resemblance of the *Shh Ihh*, *Smo*, and *Disp1*^{icb} phenotypes suggests that Disp1 is required for the spread of both Shh and Ihh ligands [4].

The Shh expression pattern revealed an interesting difference between the *Disp1*^{icb} and the *Smo*^{bnb} mutant phenotypes: the notochord in *Disp1*^{icb} was intact and expressed high levels of Shh protein, whereas the notochord in *Smo*^{bnb} was small and expressed lower levels of Shh (Figure 6). The notochord begins to degenerate at e9.0 in *Shh* mutants [3], similar to what we observed in *Smo*^{bnb} embryos. Thus, Shh signaling is required, directly or indirectly, for maintenance of Shh expression in the notochord. The robust notochord of e9.0 *Disp1*^{icb} mutants suggests that Disp1 is not required for Shh activity within the notochord. Similarly, *Drosophila* Hh produced in posterior compartment wing cells can activate the signaling pathway locally, but not at a distance, in the absence of *disp* [14]. Together, the results suggest that Disp1 is not required for juxtacrine signaling by Shh and is specifically required for release of Shh to an

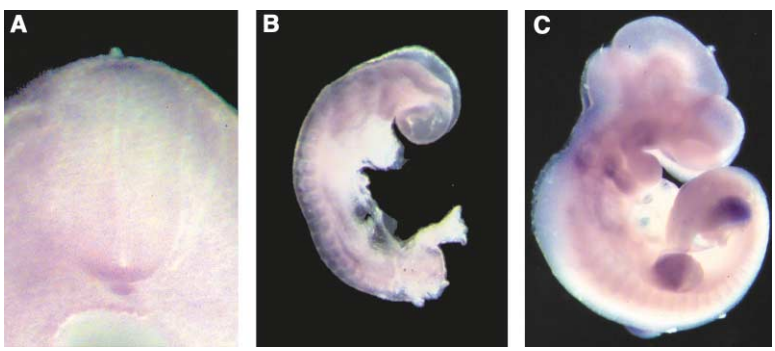

Figure 4. Localized *Disp1* Expression

(A–C) In situ hybridization showing *Disp1* expression in (A) sectioned wild-type e9.5, (B) whole-mount wild-type e9.0 (7.5× magnification), and (C) whole-mount wild-type e10.5 (5× magnification) embryos. Notochord and ventral neural tube expression of *Disp1* is clear at e9.5. At e9.0, expression is strong in the somites, branchial arches, limb buds, and neural tube. Limb bud expression becomes localized to the anterior half of the forelimb bud by e10.5.

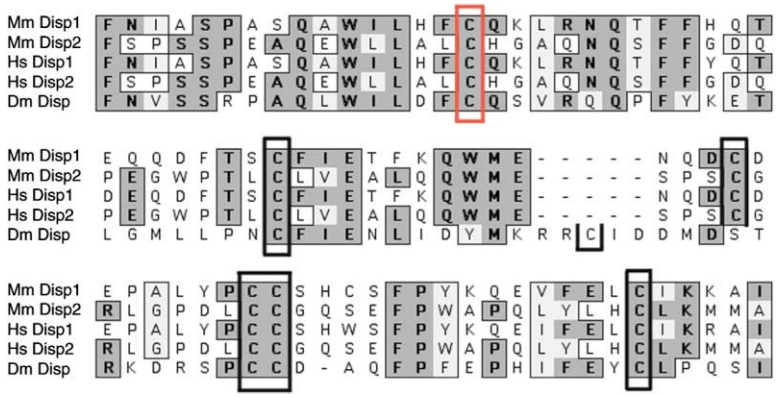

Figure 5. Alignment of the Protein Sequence Adjacent to C829F of *Disp1* with Other *disp* Family Members

C829 (boxed in red) is the first of the six conserved cysteines (others are boxed in blue) in the predicted extracellular loop. The amino acid residues that are shown are as follows: Mm Disp1 (814–892), Mm Disp2 (561–639), Hs Disp1 (816–894), Hs Disp2 (561–639), and Dm Disp (766–848).

extracellular compartment from which Shh can move to more distant cells. Long-range signaling by Shh is important for patterning the mouse neural tube, somites, and limb bud. In contrast to the case in *Drosophila*, where *disp* is expressed ubiquitously, localized expression of *Disp1* in the mouse embryo could play a decisive role in determining where Shh can act at a distance.

Experimental Procedures

Genetic Crosses and Identification of Mutant Lines

A total of 242 lines were established from individual F1 male progeny of ENU-treated males. G3 embryos that were potentially homozygous for newly induced mutations were examined for abnormal morphology at e9.5, as previously described [2]. A total of 36 mutations were identified, including the 6 that affected ventral neural tube patterning (http://mouse.ski.mskcc.org/). Approximately 1 out of 40 lines tested had a mutation that affected specification of ventral neural cell fates and left-right asymmetry. Assuming that 50 new mutations were induced per line [22], then approximately 1 out of 2000 induced mutations fell into this class. All mutations were inherited as recessive Mendelian traits for at least three generations after initial identification and produced highly or completely penetrant phenotypes in homozygous embryos. Sperm from mutant lines was cryopreserved.

Mapping

New mutations were induced on a C57BL/6 background, and all outcrosses were with C3H mice, as described previously [2]. Linkage of the mutant phenotype to C57BL/6 alleles of SSLP polymorphisms was used for gene mapping. Genome scans with DNA from approximately 24 tested carrier adults was sufficient to detect 1–4 regions of possible linkage, with polymorphic markers spaced at 20–40 cM. Mutations were mapped using a genome scan with a selected set of mapped SSLPs that could be assayed on ethidium-stained agarose gels [23]. Once intervals of potential linkage were identified, additional carriers and embryos were used to confirm linkage and refine the map position.

All mice and embryos were genotyped as described [24].

Molecular Marker Analysis

In situ hybridization and immunohistochemical staining were carried out as described previously [24].

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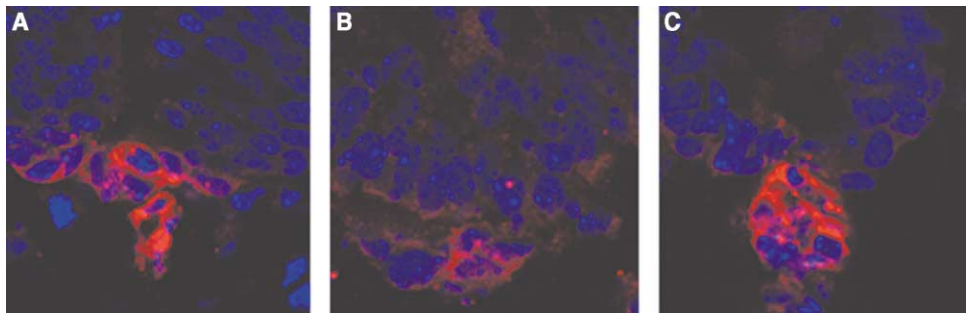

Figure 6. *Disp1* Is Required for Shh in the Neural Tube but Not for Maintenance of Shh Expression in the Notochord

(A–C) Immunohistochemical staining for Shh (red) counterstained with DAPI (blue) in sections of the notochord and ventral spinal cord of e9.5 embryos, which are visualized by confocal microscopy at 63× magnification. The three photos are taken at identical exposure settings. (A) Wild-type, (B) Smo^{bnb}, and (C) $Disp1^{icb}$ embryos. Shh is present in both the notochord and floor plate of (A) wild-type embryos. Only background levels of Shh are detected in (B) Smo^{bnb} at e9.5, although Shh expression in the notochord is initially established via an Hnf3β-dependent mechanism at e8.5 [25]. Shh is expressed at normal levels in the notochord of (C) $Disp1^{icb}$ embryos, but no Shh is detectable in the spinal cord.

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Accession Numbers

The GenBank accession number for the sequence reported in this paper is AY144589.